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ABSTRACT

Commonly known as Holy basil, *Ocimum tenuiflorum* (Lamiaceae), is a popular medicinal herb used for treating ailments ranging from colds to chronic diseases, such as cancers and diabetes. While Holy basil is well known in India and Southeast Asia, the plant is less known in western countries where a lack of information on cultural practices exists. In the current study, a field trial was established to determine the optimum planting date, the changes in essential oil content, and composition of *O. tenuiflorum* in Alabama. A total of three Holy basil accessions, PI 652056, PI 652057, and PI 288779, were planted three times at monthly intervals, beginning in April 2007. At 30, 60, and 90 days after transplanting (DATP), the aerial parts of two plants from each plot were harvested and used to determine essential oil content and composition of stems and leaves using gas chromatography-mass spectrometry. Sensory properties, yield, and composition of essential oils were affected by planting dates and harvest times. Harvesting during summer months yielded the highest amounts of oil for all three accessions. The chemotypes were identified as one high in eugenol (PI 652056), one high in β-caryophyllene (PI 652057), and a third dominated by eugenol (PI 288779) at the end of the growing period. In accession PI 652056, the level of eugenol increased with a delay in harvest time. For accession PI 652057, the level of β-caryophyllene was high at the 30-day harvest, but decreased significantly by the time of the 60-day harvest when eugenol became the dominant essential oil constituent. For the third accession (PI 288779), the essential oil was dominated by eugenol, reaching over 50% eugenol at 60 days DATP in the June planting, but the percentage of eugenol decreased towards the end of the growing season with a significant increase in (trans)-β-guaiene by 90-DATP. Of the 26 essential oil components identified in the accessions, eugenol, β-caryophyllene, E-methyl cinnamate and (trans)-β-guaiene were the most abundant constituents. The level of these essential oil constituents varied significantly in all accessions at all harvest stages. For *O. tenuiflorum* seedlings, the date of seeding, transplanting, and harvest DATP (plant maturity) significantly impacted total essential oil content and composition, although the level of changes within the various constituents were dependent upon the accession.

INTRODUCTION

Basil (*Ocimum* spp.) species, which have been used for centuries as spices and medicinal plants, contain essential oils, responsible for characteristic aromas, and other non-volatile components, such as rosmarinic acid (Koroch et al., 2010; Vieira and Simon, 2000). *Ocimum tenuiflorum* L., formerly classified as *O. sanctum* L. and commonly referred to as Holy basil, has been used historically and currently as a medicinal herb for a myriad of ailments and diseases (NIIR, 2005). Similar to other members of the *Ocimum* spp., the basil, *O. tenuiflorum*, has several chemotypes, morphologically indistinguish-able, but differing in chemical constituents. The aromatic oil of this species possesses a characteristic, pleasant aroma with an appreciable note of cloves and sometimes licorice.

The chemical composition of the oil of *O. tenuiflorum* (Holy basil) has been extensively reviewed and previously described (Lawrence *et al*., 1972; Lawrence *et al*., 1980; Malik *et al*., 1986; Laakso et al., 1990; Pushpangadan and Brada, 1995; Pino *et al*., 1994; Machado *et al*., 1999 and Maheshwari *et al*., 1987), but in contrast to the popular culinary sweet basil (*O. basilicum* L.), little is known about the best production practices for Holy basil. As a crop, Holy basil has been in cultivation in the U.S.A. for only a short time, limiting the number of cultural studies. Similar to many other aromatic plants of the same species, different accessions of Holy basil could be expected to produce essential oils with differences in chemical composition (Juliani et al., 2002; 2008; 2011; Putievsky et al., 1999).

Several environmental factors and harvest times have been observed to affect the chemical content and composition of essential oil (Juliani et al., 1994). Understanding the development of oil production by Holy basil accessions subjected to cultivated conditions could lead to optimization of oil yields, consistent oil quality, and the production of standardized, dry botanical preparations (Franz, 1983; Palevitch, 1991). The objective of the current study was to assess the essential oil content and composition of Holy basil as affected by dates of planting and harvesting.

MATERIALS AND METHODS

Plant material. Holy basil (*Ocimum tenuiflorum* L., family Lamiaceae) accessions (PI 652056, PI 652057, and PI 288779) were used in this study. Seeds of all three accessions, obtained from USDA North Central Regional Plant Introduction Station (NC7), Ames, IA, were seeded in germination trays filled with a soilless potting mix (Pro-Mix) on February 15, March 15, and April 15. After seeding, the trays were placed in a glass greenhouse at Alabama A&M University, Normal, AL (natural daylight increasing from 11 h in mid-March to about 14.5 h in early June; mean air temperature maintained at 26°C) for 45 days for germination and seedling development. The seedlings were subsequently transplanted into the field in April, May, and June, respectively.

The seedlings were placed into separate raised beds (50 cm wide, 15 cm high, 25 m long, 2 m apart, covered with 4 mm black plastic with drip irrigation tubing underneath the plastic). The raised beds were located in an experimental field at the Alabama A&M Winfred Thomas Agricultural Research Station located in Hazelgreen, AL (latitude 34°89'N and longitude 86°56'W). Soil at the experimental site was a Decatur silt loam (fine, kaolinitic, thermic Rhodic Paleudult).

Sampling and growth analysis. In each accession plot for each planting date, two randomly selected plants of each accession from each planting were harvested at 30 day intervals after transplanting into the field. The harvested plants were separated into leaves, stems (including branches and inflor-escences) and roots. The separated plant parts were weighed and then dried to a constant weight in a forced air drying oven at 21^{o}C.

Due to a lack of maturity in the transplanted plants, only those plants transplanted in April, were suitable for harvest and separation into parts at 120 days after transplanting. The shorter growing season of the transplantings in May and June could only be sampled at 30, 60, and 90 days after the transplanting for extraction of essential oils and chemical profiling. The harvest times corresponded to early vegetative, early flowering, and seed forming growth stages of the plants. Plants in accession PI 652056 from the May planting only had adequate shoot (leaf and stem)

growth at 30 DATP for essential oil extraction and constituent determination.

Extraction and analysis of essential oil. Representative plant samples were separated by planting dates and harvest times for extraction of essential oil by hydrodistillation using a Clevenger apparatus. The plant material (25 g of dried leaf and stem tissue) was placed into a 2 L distillation flask with 1 L of distilled water and boiled for 2 h to distill oil. The collected oil was allowed to cool, and then the oil volume was measured and characterized for color and cloudiness, before being transferred into amber vials. The vials with oil were stored in a freezer at -20°C until analyzed by GC and GC/MS to determine oil constituents. The oil content was expressed on a dry tissue weight basis.

For gas chromatography, the extracted oil samples were diluted (10 µL of oil in 1 mL methyl tert-butyl ether) and then analyzed using an Agilent 6890 Series FID, GC System equipped with an Agilent DB-5 bonded fused silica capillary column (5% phenyl/95% methyl silicone; 30 m x 0.25 mm i.d.). Helium was used as the carrier gas at a column pressure of 16.2 psi (0.112 MPa). The oven temperature was held at 60°C for 2 min and then programmed to increase at 4°C/min to 200°C, split ratio of 50:1 at a flow rate of 1 mL min^{-1}. The injector temperature and detector temperatures were 180°C and 220°C, respectively. Separation and identification of known samples of oil constituents (*p*-cymene, 1,8-cineole, terpinen-4-ol, methyl chavicol, geraniol, thymol, eugenol, methyl eugenol and β-caryophyllene) were used to confirm constituent separation.

Identification of the essential oil constituents was confirmed using the described Agilent 6890 GC and conditions connected to a 5973 Agilent Network Mass Selective Detector and a split ratio of 25:1. Constituent identifications were accomplished using retention times, co-injection with authentic standards where possible, and matching the mass spectra with standards from MS compound libraries (Wiley 275.L). Retention indices for n-alkanes series (8 through 20) were calculated to provide additional peak confirmation identity by comparing RI values with those of the literature (Adams, 1995).

RESULTS

Essential oil characterization. The essential oils extracted from accession PI 652056 at different harvest dates ranged in color from yellow to colorless, although some samples had tints of green color, and oil from the 60 days after transplanting harvest in June, had a brown coloration (Table 1). Some cloudiness appeared in the oils near the end of the distillation period. The highest oil level in tissue was in the May planting at the 60 DATP harvest. The lowest oil contents (0.4%) were in the 30 DATP, regardless of the planting date.

Table 1. Sensory properties and oil yield for accession PI 652056.

Transplanting date (month)	Harvest date (DATP)	Essential oil	
		Appearance[1] (color)	Yield[2] (% of dry wt.)
April	30	Y to C	0.4
	60	LY	0.4
	90	LGY	0.6
	120	LY	0.4
May	30	Y to G	0.4
	60	Y to G	0.7
	90	MC	0.4
June	30	GY	0.4
	60	LY to B	0.9
	90	C to GY	0.5

[1]Key: Y=yellow, LY=light yellow, LGY= light greenish yellow, GY=greenish yellow, B=brown, C=colorless, MC=Mostly colorless. All oils were clear, except for the April 60 day that had a few cloudy samples and the May 60 day that had some limited cloudiness.

[2]Calculated % oil = mL of oil in 100 g dried plant tissue.

Essential oil production by accession PI 652057 primarily had a green to yellowish colored oil at all harvest dates (Table 2). All the oils were clear except for the 60 DATP in April, in which the oil was cloudy, and the harvest at 90 DATP in June, in which some cloudiness was apparent. The lowest oil pro-duction was associated with the harvest of April planting at 30, 60, and 120 DATP. The highest oil yield for the accession PI 652057 was 0.9% from the June planting at 60 DATP.

Accession PI 288779 produced essential oils with a greenish yellow color and a tendency towards lighter colors at the end of each growing period (Table 3). The oils were generally clear, although some of the replications in the April planting were cloudy. The oil

yields were lowest at 30 DATP in the May and June plantings. The highest level of essential oil was from the June plantings at 60 DATP.

Table 2. Sensory properties and oil yield for accession PI 652057.

Transplanting date (month)	Harvest date (DATP)	Essential oil	
		Appearance[1] (color)	Yield[2] (% of dry wt)
April	30	GY	0.6
	60	GY	0.6
	90	GY	0.8
	120	GY to LY	0.6
May	30	GY	0.8
	60	GY	0.7
	90	GY to LY	0.7
June	30	LGY	0.8
	60	GY	0.9
	90	GY	0.8

[1]Key: Y=yellow, LY=light yellow, LGY= light greenish yellow, GY= greenish yellow, B=brown, C=colorless, MC=mostly colorless. All oils were clear, except for the April 60 day that were cloudy, and the June 90 that had some cloudiness.

[2]Calculated % oil = mL of oil in 100 g dried plant tissue.

Table 3. Sensory properties and oil yield for accession PI 288779.

Transplanting date (month)	Harvest date (DATP)	Essential oil	
		Appearance[1] (color)	Yield[2] (% of dry wt)
April	30	GY	0.8
	60	GY	0.8
	90	GY	1.2
	120	LY to GY	0.8
May	30	GY	0.6
	60	GY	0.9
	90	LY	0.7
June	30	LGY	0.6
	60	GY	1.0
	90	LY	0.8

[1]Key: Y=yellow, LY=light yellow, LGY= light greenish yellow, GY=greenish yellow, B=brown, C=colorless, MC=mostly colorless. All oils were clear, except for the April 30 day that was cloudy, the April 90 & April 120 that had few cloudy, and the June 90 that had some cloudiness.

[2]Calculated % oil = mL of oil in 100 g dried plant tissue.

Essential oil constituents. The essential oil constituents varied with the accession, the transplanting date, and the DATP harvest time. For example, an analysis of essential oil in accession PI 652056 included a number of major oil constituents within the oil profile (eugenol @ 25.3% to 51.5%, β-caryophyllene @1.2% to 25.4% and trans-β-guaiene @ 9.4% to 19.2%) (Table 4). Minor oil constituents of accession PI 652056, including (E)-α-bergamotene (@ 1.1% to 2.8%), caryophyllene oxide (@ 1.7% to 3.9%), 1,8-cineole (@ 2.2% to 9.2%), E-methyl cinnamate (@ 0.1% to 8.7%), 1,10 di-epi-cubenol (@1.3% to 2.6%), and trans-β-farnesene (@ 1.6 to 4.1%), also varied with the transplanting and harvest dates, but differences were generally quite limited due to the small percentage of these constituents in the essential oil. A total 24 oil constituents were identified in PI 652056

Similar changes in constituent levels associated with transplanting date and harvest date were noted in major oil constituents (β-caryophyllene and eugenol) in accession PI 652057. In this accession, β-caryophyllene was the major constituent, ranging from 35.1 to 49.9% of the essential oil (Figure 1). The amount of β-caryophyllene, however, decreased at later harvest dates for all transplanting dates. The accessions transplanted in April and harvested 120 days later, had the lowest levels of β-caryophyllene (data not shown). The level of eugenol (a second major constituent) in accession PI 652057 was highest at 60 DATP from the June transplanting date, but significantly decreased by 90 DATP (Figure 2).

Other essential oil constituents identified from all harvests and transplanting dates for accession PI 652057 included E-methyl cinnamate, β-selinene, bicyclogermacrene, and trans-β-guaiene (data not shown).

In PI 288779, β-caryophyllene was the major constituent, ranging from 21.2% to 40.1% of the essential oil (Figure 3). The maximum amount of β-caryophyllene was observed at 30 DATP in plants transplanted in June. By 60 DATP, the level of β-caryophyllene in plants transplanted in June and the other months had significantly decreased as com-pared with the levels at 30 DATP for the three transplanting dates of April, May, and June. In contrast, the level of eugenol showed an opposite trend with higher concentrations at 60 DATP as compared with the 30 DATP (Figure 4). This trend of increasing levels of eugenol in accession PI 288779 was observed for the April, May and June transplanting times.

Table 4. Chemical composition of essential oil of *O. tenuiflorum* accession PI 652056.

RI[1]	Essential oil constituent	Transplanting dateDATP[2]						
		April		May			June	
		60	90	30	60	90	60	90
		(Relative percentage of total volatile oil)[3]						
858	(Z)-3-hexanol	8.1 ±2.6	1.8 ± 1.5	1.0 ± 0.5	0.6 ± 0.3	0.5 ± 0.1	0.6 ± 0.0	2.5 ± 1.0
981	β-pinene	--[4]	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	--	0.1 ± 0.0	0.1 ± 0.1
1033	Limonene	--	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	--	0.1 ± 0.0	--
1036	1,8-cineole	2.2 ± 0.6	9.6 ± 1.7	9.2 ± 1.8	8.2 ± 4.2	--	5.9 ± 0.1	8.7 ± 2.2
1051	(E)-β-ocimene	--	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.3	--	0.3 ± 0.0	0.4 ± 0.1
1100	Linalool	0.7 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.4 ± 0.0	0.6 ± 0.1
1171	Borneol	0.2 ± 0.1	0..4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.1	0.3 ± 0.0	0.4 ± 0.0
1193	α-terpineol	1.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.1	0.7 ± 0.4	--	1.2 ± 0.0	1.1 ± 0.0
1254	Geraniol	--	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	--	0.3 ± 0.0	0.1 ± 0.1
1360	Eugenol	25.3 ± 3.1	30.4 ± 4.6	28.9 ± 3.7	33.5 ± 6.9	32.4 ± 5.2	51.5 ± 1.6	31.2 ± 0.7
1395	E-methyl cinnamate	0.3 ± 0.2	1.5 ± 0.1	0.4 ± 0.1	3.2 ± 3.1	8.7 ± 0.8	0.1 ± 0.0	1.0 ± 0.2
1425	(β)-caryophyllene	2.8 ± 0.2	5.0 ± 0.6	3.0 ± 0.0	15.9 ± 14.7	25.4 ± 1.4	1.2 ± 0.0	4.2 ± 0.6
1440	(E)-α-bergamotene	2.8 ± 0.2	2.0 ± 0.3	2.4 ± 0.1	1.1 ± 0.6	--	1.6 ± 0.0	2.1 ± 0.0
1447	α-humulene	0.9 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.4 ± 0.2	--	0.5 ± 0.0	0.6 ± 0.0
1460	(trans)-β-farnesene	3.5 ± 0.4	2.3 ± 0.4	4.1 ± 0.3	2.6 ± 0.3	1.6 ± 0.1	2.2 ± 0.1	2.3 ± 0.1
1486	germacrene D	0.8 ± 0.1	0.3 ±± 0.1	1.1 ± 0.1	0.3 ± 0.2	2.6 ± 0.3	0.5 ± 0.1	0.5 ± 0.0
1491	β-selinene	--	0.3 ± 0.1	--	1.4 ± 1.4	5.0 ± 0.6	--	0.2 ± 0.1
1499	bicyclogermacrene	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	1.7 ± 1.7	6.3 ± 0.7	--	0.3 ± 0.1
1511	(trans)-β-guaiene	19.2 ± 1.4	14.4 ± 1.9	17.3 ± 0.9	9.4 ± 3.3	2.6 ± 0.3	10.9 ± 0.2	15.9 ± 0.4
1527	δ-cadinene	0.3 ± 0.2	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	1.0 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
1546	α-cadinene	10.6 ± 0.9	6.6 ± 0.7	10.8 ± 0.4	4.6 ± 2.3	0.1 ± 0.1	7.3 ± 0.3	7.2 ± 0.4
1588	caryophyllene oxide	1.8 ± 0.3	2.3 ± 0.2	1.7 ± 0.1	1.9 ± 0.2	3.9 ± 0.5	1.3 ± 0.1	2.4 ± 0.0
1615	1,10 di-epi-cubenol	1.8 ± 0.3	2.6 ± 0.1	1.8 ± 0.0	1.3 ± 0.6	0.2 ± 0.0	1.7 ± 0.1	2.1 ± 0.0
1648	epi-α-cadinol	0.2 ± 0.1	0.3 ± 0.1	--	0.1 ± 0.1	--	0.2 ± 0.0	0.2 ± 0.0

[1]Retention index for indicated essential oil constituents.

[2]Plants were planted in indicated months and harvested for essential oil analysis after 30, 60, and 90 days after transplanting.

[3]Means ± S.D. of three replicate samples.

[4]No tissue available for oil extraction.

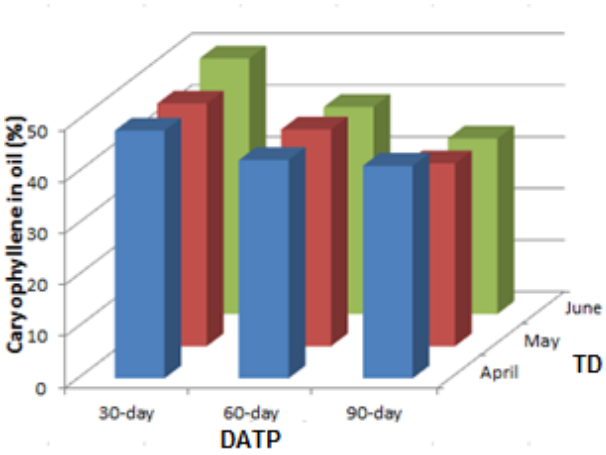

Figure 1. Changes in β-caryophyllene levels associated with transplanting date (TD) and harvest time (DATP) for Holy basil accession PI 652057.

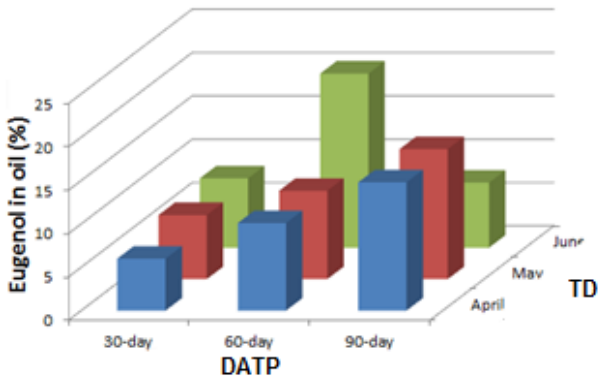

Figure 2. Changes in eugenol associated with transplanting date (TD) and harvest time (DATP) for Holy basil accession PI 652057.

In PI 288779, β-caryophyllene was also the major constituent at 21.2% to 40.1% of essential oil (Figure 3). The highest amount of β-caryophyllene was observed in plants that were one month old (30-42%) for the three transplanting dates of April, May, and June, while eugenol showed an opposite trend with higher percentages at 60 DATP (Figure 4). The decrease in β-caryophyllene (from 30% - 25%) was coupled with significant increments of eugenol (from 24% - 39%). Lowest levels of β-caryophyllene (20% - 26%) were observed in all plants harvested after 90 days of transplanting (data not shown).

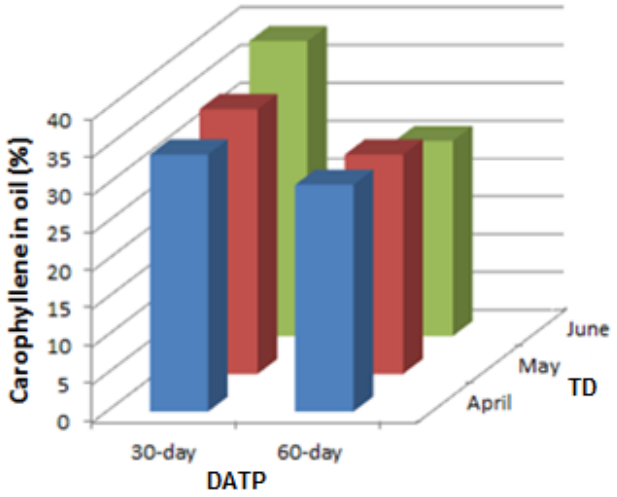

Figure 3. Changes in β-caryophyllene as transplanting date (TD) and harvest time (DATP) for Holy basil accession PI 288779.

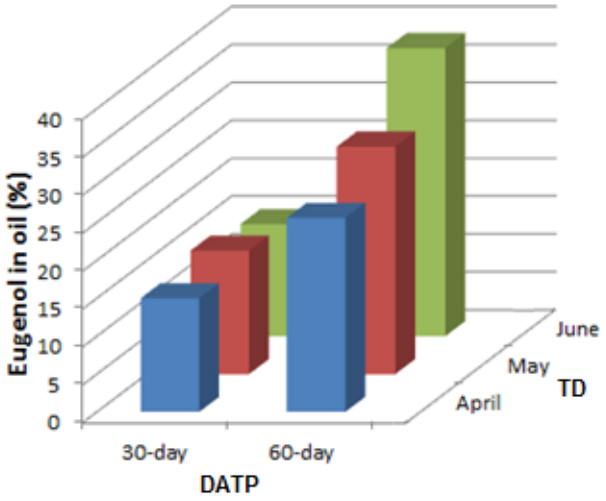

Figure 4. Changes in eugenol associated with transplanting date (TD) and harvest time (DATP) for Holy basil accession PI 288779.

DISCUSSION

Earlier studies by Simon and Reiss-Bubenheim (1992) and by Kothari et al. (2004) with sweet basil and Holy basil, respectively, demonstrated that the growth environment could affect the essential oil production. Our study, in which three accessions of Holy basil plants were subjected to different environments and harvest dates, showed an interaction of the growth environment and accession led changes in oil quality, constituency, and yield. While the accessions were exposed to the same field

environment, the essential oils differed in color, cloudiness, constituents, and yield.

Accessions PI 652056 and PI 650257 produced the highest levels of oil when transplanted into the field in June and harvested at 60 days after transplanting, a time that coincided with the relatively high temperatures observed in Alabama during the warm summer months of July and August (Anonymous, 2014). In contrast, accession PI 288779 produced relatively high levels oil throughout all transplanting and harvest dates, suggesting this accession may be less sensitive to warm temperatures. In an earlier study with *Lippia juneliana*, higher oil yields were associated with warmer summer temperatures (Juliani et al., 1998).

The chemical composition of the essential oils in this study suggested the presence of two chemotypes within the three accessions of Holy basil, one rich in eugenol (PI 652056), a second one rich in β-caryophyllene (PI 652057), and a third accession (PI 288779) similar to PI 652056, dominated by eugenol at the end of growing period. Eugenol, β-caryophyllene, methyleugenol, and (*E*)-cinnamyl acetate were the predominant essential oil constituents in this study These oil constituents were also pre-dominant in Holy basil plants grown under supplemental irrigation as opposed to plants grown under dry, semi-arid conditions (Kothari *et al*., 2004).

The amount of oils varied significantly with time as there were differences between harvests at 30, 60 and 90 DATP and also from planting date to planting date. A similar variation in essential oil content with time of harvest was reported by Kothari *et al*. (2004). In an earlier report, Kelm and Nair (1998) observed that eugenol was the major essential oil constituent at 30-70% in Holy basil. Vieira and Simon (2000) have reported volatile oil profiles rich in methyl (E) cinnamate in cultivars of several different *Ocimum* species, further demonstrating the importance of genetics as well as the environment on essential oil composition. Gill and Randhawa (1996) observed that a delay in planting date increased the linalool content and decreased the methyl chavicol and eugenol content. In this study, any decrease in eugenol and β-caryophyllene levels associated with transplanting date and harvest dates was dependent upon the accession. An observed increase in concentration of linalool, methyl chavicol, and eugenol in the essential oil is most likely due to an accession response to environment, especially longer days that would allow time for greater accumulation of constituents.

The effect of the growth environment on essential oil yield and constituents within basil species, however, remains contradictory. Bowes and Zheljazkov (2004) reported that the composition of essential oil of two sweet basil and Holy basil cultivars was not significantly altered by differences in planting dates. Simon and Reiss-Bubenheim (1992) showed that sweet basil subjected to mild and moderate water stress increased essential oil content and altered oil composition. Adler *et al*. (1989) observed that the form of nitrogen used in fertilizing sweet basil could alter the essential oil content and composition.

Dey and Choudhuri (1983) reported that the level of eugenol and methyl eugenol decline in Holy basil with progressive maturation of the plant leaves. In our study, however, essential oil content increased with leaf maturation and declined as the leaves senesced. An earlier study, with *O. gratissimum*, observed that essential oil content and the concentration of geraniol in the oil increased with leaf development and expan-sion until leaves were fully developed, but then decreased with further maturation and senescence (Charles and Simon, 1993). This change in the essential oil with leaf maturation is probably due to the higher density of oil glands on young basil leaves. Young basil leaves have been shown to have a higher density of oil glands than older leaves (Gang *et al*., 2001).

The Holy basil accessions tested in our study demonstrated that interactions of the plant environment with genetic differences among accessions within a species can lead to changes in essential oil production and constituents. The development agricultural practices that can lead to high levels of productivity with the desired essential oil constituents will require additional testing within defined environments to best develop the base-line cultural practices and those

accessions that best fit environments. While environmentally induced variation in natural products are often overlooked, this variation can significantly affect production and quality of the essential oil.

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